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Spatial distribution of metals within the liver acinus and their perturbation by PCB126

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Abstract

Animal studies show that exposure to the environmental pollutant 3,3',4,4',5-pentachlorobiphenyl (PCB126) causes alterations in hepatic metals as measured in acid-digested volume adjusted tissue. These studies lack the detail of the spatial distribution within the liver. Here we use X-ray fluorescence microscopy (XFM) to assess the spatial distribution of trace elements within liver tissue. Liver samples from male Sprague-Dawley rats, treated either with vehicle or PCB126, were formalin fixed and paraffin embedded. Serial sections were prepared for traditional H&E staining or placed on silicon nitride windows for XFM. With XFM, metal gradients between the portal triad and the central vein were seen, especially with copper and iron. These gradients change with exposure to PCB126, even reverse. This is the first report of how micronutrients vary spatially within the liver, and how they change in response to toxicant exposure. In addition, high concentrations of zinc clusters were discovered in the extracellular space. PCB126-treatment did not affect their presence, but did alter their elemental makeup suggesting a more general biological function. Further work is needed to properly evaluate the gradients and their alterations as well as classify the zinc clusters to determine their role in liver function and zinc homeostasis.

Keywords

PCBs; liver; metal homeostasis; XFM; copper; iron

Introduction

The liver is a dynamic organ with a host of different functions, including xenobiotic metabolism and serum protein synthesis, as well as storage capabilities, in particular, the storage of micronutrients and trace elements (Casarett & Doull 2013). The majority of

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Supporting Information

Figure S1. H&E serial section and XFM micrographs for phosphorus and sulfur in livers of vehicle and PCB126 treated rats (PDF). Figure S2. Quantification of element distribution and its change relative to distance from the portal triad in livers of vehicle and PCB126 treated rats (PDF). Figure S3. Zinc distribution across the liver acini and the corresponding H&E serial section (PDF).

studies investigating the storage function of the liver have described micronutrient homeostasis at the whole-organ level using volume-averaged analytical techniques. (Klaren et al. 2015, Lai et al. 2010). However, few studies have investigated the spatial micronutrient distribution within the liver at the functional unit level, the liver acinus. Given that gradients are known to exist along the liver acinus, e.g. oxygen tension, a better understanding of whether other aspects of liver function are present as gradients is needed.

Polychlorinated biphenyls (PCBs) were once widely used industrial chemicals whose chemical inertness allowed for their use in a variety of different applications ranging from electric insulation to plasticizers (Silberhorn et al. 1990). Unfortunately, it is that chemical property that allowed PCBs to persist in the environment and bioaccumulate. PCBs have been found in remote areas, but especially in urban settings where sources with high levels of PCBs and high airborne levels can be found (Ampleman et al. 2015, Grimm et al. 2015). PCBs consist of 209 different chemical congeners which can be subdivided into their structural properties and the biological effects they exert. A well-studied group of PCBs are the dioxin-like PCBs which act in a similar manner as TCDD (2,3,7,8-tetrachlorodibenzodioxin) via activation of the aryl- hydrocarbon receptor (Gadupudi et al. 2016).

A less understood biological effect of dioxin-like compounds, e.g. 3,3',4,4',5-pentachlorobiphenyl (PCB126), is alterations in hepatic trace elements upon chemical exposure (Lai et al. 2010, Lai et al. 2013, Lai et al. 2011). A recent investigation demonstrated that the micronutrient disruption that occurs follows a change in hepatocyte architecture, as reflected in lipid accumulation (Klaren et al. 2015). The traditional means of quantifying trace elements, volume averaged quantitation by acid-digested liver tissue using ICP-MS, used in that study was unable to link the change in hepatocyte vacuolation spatially with the micronutrient disruption. To better understand the cause of hepatic micronutrient alteration, an exploration of the spatial aspects of the liver damage and the trace elements was needed.

X-ray Fluorescence Microscopy (XFM) is a powerful technique that allows for the determination of the elemental 2D spatial distribution in a sample (Fahrni 2007). A crucial aspect of this technique is the high resolution that is attainable which yields fine structural analysis in addition to elemental composition. The technique is based fundamentally on x-ray induced fluorescence (XRF) from atoms in the sample. The energy of the emitted photon is characteristic of the element from which it was emitted. The sample is raster-scanned through a focused hard x-ray beam (10 keV), and at each scan position a full XRF spectrum is acquired thus allowing for quantification of the elements within the sample.

Tissues, including liver and kidney, have been analyzed with XFM in the past while investigating a range of elements but until now the elemental distribution of the liver has not been assessed at the level of the functional unit, the acinus (Delfino et al. 2011, Malinouski et al. 2012, Ralle et al. 2010, Weekley et al. 2014, Yuxi et al. 2005). This study uses XFM with a large field of view, at high resolution to determine the micronutrient gradient and microenvironments established in the liver and also its perturbation by PCB126. Novel

findings regarding extracellular localization of higher concentrations of zinc which may play a role in zinc homeostasis were also discovered and presented here

Experimental

Chemicals

All chemicals, unless otherwise noted, were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). The synthesis of PCB126 was carried out with an improved Suzuki-coupling method using 3,4-dichlorophenyl boronic acid and 3,4,5-trichlorobenzene as reactants and a palladium catalyst for the cross coupling reaction (Luthe et al. 2006). Purification was conducted using an aluminum oxide column and flash silica gel column chromatography and methanol recrystallization. The purity of final product >99.8% assessed by GC/MS and structure confirmed by ^{13}C NMR.

Animal Care

University of Iowa Institutional Animal Care and Use Committee approval was obtained for the following animal study. Five male Sprague Dawley rats (75–100 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN). The rats were housed individually in wire hanging cages, to monitor feed consumption, in a 12 hour light-dark cycle and constant temperature and humidity. The 4–5 week old animals were fed, *ad libitum*, a purified AIN-93G diet, purchased from Harlan Tekland (Madison, WI), for three weeks to allow for hepatic trace element equilibration. The animals were then given a single intraperitoneal injection of either tocopherol-stripped soy oil (Harlan Tekland (Madison, WI)), at 5 ml/kg (n=3), or 5 $\mu\text{mol/kg}$ PCB126 dissolved in tocopherol-stripped soy oil (n=2). After two weeks of exposure, the animals were sacrificed by CO_2 asphyxiation followed by thoracotomy. Organs were collected for further analysis.

Sample Preparation

Pieces of liver were placed in 10% neutral buffered formalin overnight before processing and embedding in paraffin. Serial sections (4 μm thick) were cut on a microtome and placed either on a silicon nitride window (100 nm membrane; 1.5 mm $\times$ 1.5 mm window), purchased from Structure Probe, Inc. (West Chester, PA), or on traditional glass slides. Glass slides were further processed and stained with Hematoxylin and Eosin (H&E) for histological examination and for intra-hepatic reference. Sections on silicon nitride windows were placed in a desiccator for additional drying.

X-ray Fluorescence Microscopy

X-ray Fluorescence microscopy was carried out on Beamline 2-ID-D/E at the Advanced Photon Source in Argonne National Labs (Argonne, IL) for the mapping of trace elements. Prior to elemental mapping, phase contrast images were acquired to find areas of interest with reference to H&E stained slides. An undulator was used to generate 10 keV x-rays for elemental mapping. The X-rays were focused to a 0.5 micron spot size using a Fresnel zone plate lens and the emitted X-ray fluorescence spectrum was detected using a 4-element silicon drift diode (Vortex-ME4, Hitachi). Samples were placed in a helium environment to reduce air-scattering background. A single bounce silicon 111 reflection was used to

monochromatize the X-ray beam. MAPS software was used to normalize and fit the x-ray fluorescence spectra at the characteristic energies for each element (Vogt 2003). Quantification of the images was conducted by fitting the sample spectrum against a thin film standard. Singular large-view scans were performed on a single section of liver from a single rat within the different treatment groups (Vehicle (shown), and 5 $\mu\text{mol/kg}$ (shown)). Six small-view scans were made within the 5 $\mu\text{mol/kg}$ PCB126 treated rat sample for assessment of zinc clusters. Resultant histological images and elemental micrographs were discussed with a board-certified veterinary pathologist for additional insight into effects.

Additional Data Analysis: Gradient Quantification

Using the MAPS software, quantitation of specific elements was determined in specified locations. Regions of interest, or ROIs, roughly 30 μm by 30 μm (the size of a hepatocyte) in size were drawn over the micrographs starting near the central vein and moving in a stepwise pattern until the portal triad was reached. Concentrations, given in $\mu\text{g}/\text{cm}^2$, for each ROI were determined to quantify the gradient of elements.

Results

By scanning the whole liver acinus at a high resolution, morphological features and their elemental composition were acquired. Key histological features were visible in the micrographs that were generated, notably the central vein and portal triad (Figure 1). These matched with the H&E stained serial sections (Figure 1). Higher abundance elements like phosphorus and sulfur seem evenly distributed throughout the liver acini with little change in concentration from the central vein to the portal triad in control livers (Figure S1). This was confirmed by quantitation (Figure S2). Upon treatment with PCB126, clear alterations in hepatocellular structure were seen both in the H&E stain and also the micrographs (Figure 1 & Figure S1). Lipid vacuolization was apparent and seen as black spots in the liver tissue. Although cytoplasmic rearrangement had occurred, the macronutrient elements appeared to remain spatially consistent, again confirmed in the quantitation (Figure S2).

The trace elements investigated differed from the more abundant elements as they appeared to have gradients (Figure 1a). This was particularly seen with iron and to a lesser extent with copper and manganese. In control livers, higher iron concentrations were seen near the central vein, gradually decreasing toward the portal triad. After quantitation, the gradients of copper and manganese were more apparent, however their gradient was the inverse of that of iron, higher concentrations near the portal triad and lower near the central vein (Figure 1c). Zinc appeared to remain stable throughout the liver acini. Visualization and quantitation of treated livers suggest a disruption of the micronutrient gradient. This was particularly seen with copper and manganese, and iron to a minor degree (Figure 1b&d).

Examination of the sinusoidal space resulted in the discovery of higher concentrations of zinc confined to a circumscribed structure. It is important to note that the presence or amount of zinc clusters was found regardless of treatment with PCB126, suggesting the changes caused by the toxicant were not involved with the zinc manifestation (Figure 1). To confirm their presence and garner a more refined image additional micrographs were acquired. These images confirmed their location in the sinusoidal space (Figures 2 & 3). In

the treated livers, lipid vacuoles were clearly seen as well as nuclei. The haze that is apparent in the iron micrograph and appears green in the tricolor image is likely cytoplasmic ferritin. The location of the zinc cluster near the border of the hepatocyte also suggests an extracellular location. The composition of the zinc cluster shows a concentration of zinc that is equal to or, at times, higher than that of iron (Table 1). In addition, the level of zinc and iron is roughly two and three times higher than the surrounding hepatic tissue, respectively. The composition of the zinc clusters was somewhat modulated by the treatment of PCB126 with the zinc to iron ratio changing from 0.83 in the liver of vehicle-treated rats to 0.42 in the liver of PCB126-treated ones.

Discussion

The dynamic nature of the liver gives rise to gradients, the most well-known of which is oxygen tension. The presence of other gradients, especially those pertaining to micronutrients, is not fully known or understood. This investigation set out to investigate the presence of micronutrient gradients across the functional unit of the liver, the liver acinus. In addition, this study gauged the response of these gradients to perturbation of liver physiology by an environmental pollutant.

The more abundant elements investigated remained at roughly the same quantity throughout the liver acinus (Figure S1&S2). These elements are vitally important in normal cellular processes as well as being integral parts of proteins and nucleic acids. It is important to note that formalin fixation is known to make cells more permeable thus altering the more diffusible elements, like K and Ca (James et al. 2011, Schrag et al. 2010). Future studies investigating the elemental composition of paraffin embedded tissues may need to take this into consideration. In addition, cryofixation should be included in future work to properly investigate more diffusible elements and eliminate any potential fixation or embedding artifacts. With exposure to PCB126, the spatial distributions of these macronutrients remain unchanged which supports the essential nature of these elements (Figure S1 & S2).

Trace elements in control livers were seen as a gradient as opposed to the more abundant elements (Figure 1a & 1c). The gradients that were present are what would be expected given the physiology of the liver. Iron had higher concentrations near the central vein compared to the portal triad. Given the higher expression of cytochrome P-450 metabolizing enzymes near the central vein, an increase in iron is likely. In contrast, copper and manganese had higher levels near the portal triad. This may also be a reflection of liver function, given that both copper and manganese are secreted in the bile which collects in the bile duct. Interestingly, the gradients appear disrupted upon exposure to PCB126 (Figure 1b & 1d). This is most profoundly seen with the quantitation of copper and manganese. Previous studies have demonstrated that compounds similar to PCB126 can disrupt bile flow which could result in the disruption of the gradient of biliary secreted metals.

The presence of zinc clusters in the extracellular space was a new finding. These were present in tissue from both control and treated animals (Figure 1). Interestingly, the elemental composition appeared somewhat dependent on treatment with the alteration of the zinc to iron ratio suggesting some biological origin (Table 1). The size of the clusters is quite

large, roughly 5 μm in diameter, which eliminates any serum protein that contains a higher percentage of zinc (Figures 2 & 3). Although, erythrocytes are 6–8 μm in size, the elemental makeup of the clusters had more or equivalent levels of zinc and iron which would be highly irregular in a red blood cell that usually has roughly 40x times more iron than zinc (Table 1) (King & Cousins 2006). Other cells in the sinusoidal space might also be considered, in particular Kuepfer and stellate cells. Macrophages are reliant on zinc to properly function, however, the distribution of zinc within that cell type is unknown (Wirth et al. 1989). Future studies investigating these clusters should consider acquiring light micrographs for a direct comparison with structural features to provide more insight into the exact origin of the clusters. It is also possible that the clusters are the result of fixing, processing, and embedding in paraffin. Studies have shown that chemical fixation, although good for structural analysis, alters the distribution of elements, especially those that are more diffusible ions (James et al. 2011, Schrag et al. 2010, Twining et al. 2003). If that is the case, it would be interesting to know the source of the zinc since all solutions used would only have a trace level of zinc and would unlikely produce the size cluster that was observed. Similar to the larger full acinar scans, future studies should incorporate cryofixation as a way to further probe the more diffusible elements and further characterize these clusters.

This work demonstrates that trace elements in the liver are present as gradients within the acinar structure. This work also demonstrates that these gradients may be perturbed by exposure to a toxicant (Figure 4). In addition, hepatic micronutrient disruption previously observed with PCB126 may be an aggregate of changes occurring across the many acini in the liver. Alterations in the microenvironments within the acinus in the absence and presence of toxicant exposure supports the different zonal features that may be reflected histologically. The presence of sinusoidal zinc clusters is also new, raising multiple questions regarding their function, localization, and contribution to labile zinc pools and overall zinc homeostasis. Additional work is needed to confirm these findings especially given the small sample size. The visualization of hepatic trace element gradients, and their alteration, is novel as is the discovery of extracellular zinc clusters.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Ampleman MD, Martinez A, DeWall J, Rawn DF, Hornbuckle KC, Thorne PS. Inhalation and dietary exposure to PCBs in urban and rural cohorts via congener-specific measurements. *Environ Sci Technol*. 2015; 49:1156–64. [PubMed: 25510359]
- Casarett, LJ., Doull, J. Toxic Responses of the Liver. In: Klaassen, CD., editor. *Casarett and Doull's Toxicology: The Basic Science of Poisons*. McGraw Hill Medical; 2013. p. 557-582.
- Delfino R, Altissimo M, Menk RH, Alberti R, Klatka T, Frizzi T, Longoni A, Salome M, Tromba G, Arfelli F, Clai M, Vaccari L, Lorusso V, Tiribelli C, Pascolo L. X-ray fluorescence elemental mapping and microscopy to follow hepatic disposition of a Gd-based magnetic resonance imaging contrast agent. *Clin Exp Pharmacol Physiol*. 2011; 38:834–45. [PubMed: 21957877]
- Fahrni CJ. Biological applications of X-ray fluorescence microscopy: exploring the subcellular topography and speciation of transition metals. *Curr Opin Chem Biol*. 2007; 11:121–7. [PubMed: 17353139]
- Gadupudi GS, Klaren WD, Olivier AK, Klingelutz AJ, Robertson LW. PCB126-Induced Disruption in Gluconeogenesis and Fatty Acid Oxidation Precedes Fatty Liver in Male Rats. *Toxicol Sci*. 2016; 149:98–110. [PubMed: 26396156]
- Grimm FA, Hu D, Kania-Korwel I, Lehmler HJ, Ludewig G, Hornbuckle KC, Duffel MW, Bergman A, Robertson LW. Metabolism and metabolites of polychlorinated biphenyls. *Crit Rev Toxicol*. 2015; 45:245–72. [PubMed: 25629923]
- James SA, Myers DE, de Jonge MD, Vogt S, Ryan CG, Sexton BA, Hoobin P, Paterson D, Howard DL, Mayo SC, Altissimo M, Moorhead GF, Wilkins SW. Quantitative comparison of preparation methodologies for X-ray fluorescence microscopy of brain tissue. *Anal Bioanal Chem*. 2011; 401:853–864. [PubMed: 21533642]
- King, JC., Cousins, RJ. Zinc. In: Shils, ME, Shike, M, Ross, AC, Caballero, B., Cousins, RJ., editors. *Modern nutrition in health and disease*. Wolters Kluwer Health/Lippincott Williams & Wilkins; Philadelphia: 2006. p. 271-285.
- Klaren WD, Gadupudi GS, Wels B, Simmons DL, Olivier AK, Robertson LW. Progression of micronutrient alteration and hepatotoxicity following acute PCB126 exposure. *Toxicology*. 2015; 338:1–7. [PubMed: 26410179]
- Lai I, Chai Y, Simmons D, Luthe G, Coleman MC, Spitz D, Haschek WM, Ludewig G, Robertson LW. Acute toxicity of 3,3',4,4',5-pentachlorobiphenyl (PCB 126) in male Sprague-Dawley rats: effects on hepatic oxidative stress, glutathione and metals status. *Environ Int*. 2010; 36:918–23. [PubMed: 19969354]
- Lai I, Klaren WD, Li M, Wels B, Simmons D, Olivier AK, Haschek WM, Wang K, Ludewig G, Robertson LW. Does Dietary Copper Supplementation Enhance or Diminish PCB126 Toxicity in the Rodent Liver? *Chem. Res. Toxicol*. 2013; 26:634–644. [PubMed: 23527585]
- Lai IK, Chai Y, Simmons D, Watson WH, Tan R, Haschek WM, Wang K, Wang B, Ludewig G, Robertson LW. Dietary Selenium as a Modulator of PCB 126-Induced Hepatotoxicity in Male Sprague-Dawley Rats. *Toxicol Sci*. 2011; 124:202–14. [PubMed: 21865291]
- Luthe G, Schut BG, Aaseng JE. Monofluorinated analogues of polychlorinated biphenyls (F-PCBs): Synthesis using the Suzuki-coupling, characterization, specific properties and intended use. *Chemosphere*. 2006; 77:1242–1248. [PubMed: 16571358]
- Malinouski M, Kehr S, Finney L, Vogt S, Carlson BA, Seravalli J, Jin R, Handy DE, Park TJ, Loscalzo J, Hatfield DL, Gladyshev VN. High-resolution imaging of selenium in kidneys: a localized selenium pool associated with glutathione peroxidase 3. *Antioxid Redox Signal*. 2012; 16:185–92. [PubMed: 21854231]
- Ralle M, Huster D, Vogt S, Schirrmeister W, Burkhead JL, Capps TR, Gray L, Lai B, Maryon E, Lutsenko S. Wilson Disease at a Single Cell Level. *The Journal of Biological Chemistry*. 2010; 285:30875–30883. [PubMed: 20647314]
- Schrag M, Dickson A, Jiffry A, Kirsch D, Vinters HV, Kirsch W. The effect of formalin fixation on the levels of brain transition metals in archived samples. *Biometals*. 2010; 23:1123–1127. [PubMed: 20582563]

- Silberhorn EM, Glauert HP, Robertson LW. Carcinogenicity of polyhalogenated biphenyls: PCBs and PBBs. *Crit Rev Toxicol.* 1990; 20:440–96. [PubMed: 2165409]
- Twining BS, Baines SB, Fisher NS, Maser J, Vogt S, Jacobsen C AT-S, Sanudo-Wilhelmy SA. Quantifying trace elements in individual aquatic protist cells with a synchrotron X-ray fluorescence microprobe. *Anal. Chem.* 2003; 75:3806–3816. [PubMed: 14572047]
- Vogt S. MAPS: A set of software tools for analysis and visualization of 3D X-ray fluorescence data sets. *J. Phys. IV France.* 2003; 104:635–638.
- Weekley CM, Shanu A, Aitken JB, Vogt S, Witting PK, Harris HH. XAS and XFM studies of selenium and copper speciation and distribution in the kidneys of selenite-supplemented rats. *Metallomics.* 2014; 6:1602–15. [PubMed: 24801434]
- Wirth JJ, Fraker PJ, Kierszenbaum F. Zinc requirement for macrophage function: effect of zinc deficiency on uptake and killing of a protozoan parasite. *Immunology.* 1989; 68:114–119. [PubMed: 2680908]
- Yuxi G, Yingbin L, Chunying C, Bai L, Wei H, Yuying H, Zhifang C. Combination of synchrotron radiation X-ray fluorescence with isoelectric focusing for study of metalloprotein distribution in cytosol of hepatocellular carcinoma and surrounding normal tissues. *Journal of Analytical Atomic Spectrometry.* 2005; 20:473.

Figure 1.

Trace element distribution throughout the liver acinus and its perturbation by PCB126. (a&b) H&E serial section and XFM micrographs for iron, copper, zinc, and manganese in vehicle and PCB126 treated liver. (c&d) Quantification of element distribution and its change relative to distance from the portal triad in the liver of vehicle and PCB126 treated rats (units are $\mu\text{g}/\text{cm}^2$).

Figure 2. Discovery of extracellular zinc cluster

A tricolor image near the central vein with phosphorous (red), iron (green), and zinc (blue) indicated. Tissue from the liver of a 5 $\mu\text{mol/kg}$ PCB126 treated rat. Note the hepatocellular vacuoles and nuclei. (Bar represents 20 μm)

Figure 3. Additional extracellular zinc cluster image

A tricolor image near the central vein with phosphorous (red), iron (green), and zinc (blue) indicated. Tissue from the liver of a 5 µmol/kg PCB126 treated rat. Note the hepatocellular vacuoles and nuclei. (Bar represents 20 µm)

Figure 4.

Illustration of micronutrient distribution across the liver acinus and its perturbation by PCB126 treatment. Iron is found at higher concentrations near the central vein whereas manganese and copper are at higher concentrations near the portal triad. Upon treatment of PCB126, the gradients for manganese and copper are reversed and the extent of the gradient is diminished for iron.

Table 1
Elemental composition of extracellular zinc clusters

A total of 5 zinc clusters from each large view scan were selected for further analysis of their elemental composition. Raw values were adjusted to background by using the “empty” space (essentially paraffin) within the central vein of each image. Hepatic tissue was calculated by selecting a cytoplasmic area within a hepatocyte from each large-view scan. Averages ($\pm$ SD); Units are $\mu\text{g}/\text{cm}^2$.

	P	Fe	Zn	Ratio Zn/Fe
Vehicle Treated	0.70 ($\pm$ 0.28)	0.14 ($\pm$ 0.03)	0.11 ($\pm$ 0.04)	0.83
5 $\mu\text{mol}/\text{kg}$ Treated	0.60 ($\pm$ 0.19)	0.22 ($\pm$ 0.11)	0.09 ($\pm$ 0.02)	0.42
Average	0.82 ($\pm$ 0.30)	0.16 ($\pm$ 0.05)	0.13 ($\pm$ 0.05)	0.81
Hepatic Tissue	1.48 ($\pm$ 0.32)	0.06 ($\pm$ 0.01)	0.06 ($\pm$ 0.01)	0.89